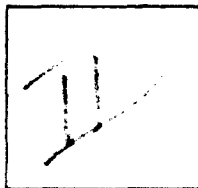


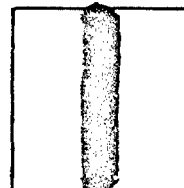
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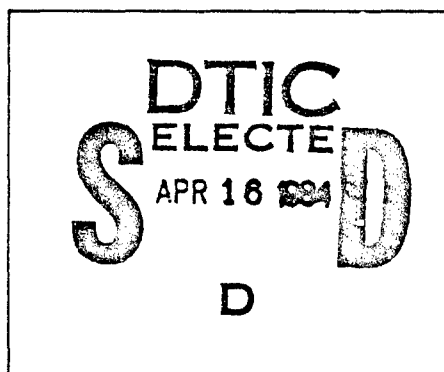
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7th Annual Report

Development of Vaccines to Prevent Wound Infections  
due to Anaerobic Bacteria

ANNUAL REPORT

Dennis L. Kasper, M.D.

August 1980

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick, Frederick, Maryland 21701

Contract #DAMD-17-78C-8019

The Peter Bent Brigham Hospital  
A Division of Affiliated Hospitals Center, Inc.  
Boston, Massachusetts 02115

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Summary of previous work  
done on this contract

In last year's annual report, a detailed summary of the extensive progress made over the last several years of this program was presented. Rather than present this entire summary again, I refer the reviewer to last year's report. This report will only review this year's progress and hopefully, the relation to the overall project. However, a few important facts which emerge from our studies need to be stated:

(1) B. fragilis is very common in infections of humans where anaerobes are likely to be found, including both bacteremia and infections with abscess formation, (2) that B. fragilis is clearly involved in the pathogenesis of experimental intraperitoneal abscesses, (3) that humans make antibody to the capsular polysaccharide of B. fragilis in natural infection and (4) that experimental infection with B. fragilis can be prevented by immunization with the capsular polysaccharide; all indicate that prevention of this disease by immunization is a feasible approach.

The studies done in the past year have concentrated on two aspects of vaccine development:

- 1) We have defined important organism factors which are involved in lot to lot variability of the capsular antigen. These variations are important in determining the reproducibility of the antigen preparation method and standards for the vaccine. Also these variations are very important to understand when doing in vitro assays of B. fragilis immunity and will be important when we test vaccine recipients for immunity.
- 2) We have also defined the mechanisms involved in immunity to experiment infection with B. fragilis. These mechanisms are extremely important to understand when determining vaccine efficacy.

### Summary of Annual Report

The amount of capsular antigen produced by Bacteroides fragilis appears to significantly decrease with in vitro passage on blood agar plates of strain 23745. This decrease in the quantity of isolatable capsule is associated with the emergence of a small transparent colony type variant (SCT). The small colony type strain also has a glycogen-like material associated with its outer membrane. This glycogen is found intracellularly, but is not associated with the outer membrane on the mucoid colony variant which predominates after animal passage, the large colony type (LCT). There is no concomitant alteration in outer membrane proteins or lipopolysaccharides associated with this colonial transformation. Electron micrographs using either ruthenium red staining or indirect ferritin antibody staining of the capsular antigen confirm the relative loss of capsule with in vitro passage. Care must be taken when doing in vitro studies of Bacteroides fragilis antigenic structure to define the degree of passage of the strain.

Neutrophil killing of the SCT and LCT bacteria was measured. With an initial inoculum of  $2.5 \times 10^7$  CFU/ml the number of bacteria surviving after 120 minutes incubation with neutrophils and human serum were  $1.37 \times 10^6$  CFU/ml for LCT and  $1.89 \times 10^5$  CFU/ml for SCT (P .01). In contrast, when B. thetaiotaomicron (unencapsulated) was studied, no significant difference in survival was noted between AP\* ( $1.47 \times 10^5$  CFU/ml) and LP\* ( $9.79 \times 10^4$  CFU/ml) bacteria (P 0.1). The addition of rabbit anticapsular antibody resulted in significantly increased neutrophil killing of both LCT and SCT B. fragilis; the number of additional bacteria killed was greater for LCT than SCT, but this difference was not statistically significant. No bactericidal activity was present when bacteria were incubated with serum in the absence of neutrophils or with heat inactivated serum and neutrophils. These results suggest that the capsule contributes to virulence by protecting the organism from neutrophil killing.

A series of experiments were designed in the model of intraabdominal sepsis to determine the cellular mechanisms of immunity induced by the capsular antigen to both the bacteremic and abscess phase of this disease. These studies which are presented in the second section of this report conclude that both cellular and humoral limbs of the immune system play important roles in protection against B. fragilis infection. As would be expected, antibody protects against bacteremia with this encapsulated organism. But the distinctive pathological marker of B. fragilis is abscess formation and these studies indicate that only T cell mediated immunity protects against this process in the experimental model.

\*AP - animal passed

\*LP - laboratory passed

## Summary of Previous Work Done on this Contract (1974-1979)

### Clinical and Microbiologic Aspects of Bacteroides

Among the diseases commonly caused by anaerobes are brain abscess, chronic sinusitis, dental infections, aspiration pneumonitis, lung abscess, empyema, liver abscess, intraabdominal sepsis, and infections of the female genital tract (1). When optimal bacteriologic techniques are used, anaerobic bacteria can be isolated from 70%-95% of patients with these diseases. A common denominator in this seemingly diverse array of septic processes is that the source of the bacteria responsible is the patient's own microflora. Thus, the anaerobic bacteria that are isolated from infected sites are normal inhabitants of the oral cavity, gastrointestinal tract, lower female genital tract, or skin. The pathogenic mechanism is usually a disruption of anatomic barriers and of other host-defense mechanisms that allows the bacteria that colonize mucocutaneous surfaces to gain access to normally sterile sites.

The bacteriology of these infections might be expected to reflect the flora of the source of the inoculum. Yet, although >400 bacterial species reside in the colon and >200 are thought to colonize healthy oral cavities, the average number of bacterial species in infections associated with colonic perforation is five (2), the average in dental infections is six (3), and for aspiration pneumonia the average is three (4). The anaerobic bacteria that dominate in these types of diseases include Bacteroides fragilis, Bacteroides melaninogenicus, Fusobacterium nucleatum, Clostridium perfringens, Peptostreptococcus anaerobius, and Peptococcus asaccharolyticus-six species that probably account for the great majority of anaerobic isolates in clinical laboratories (5). Thus, from a seemingly endless array of anaerobic bacteria in the normal flora, only a few are common in septic processes; it is likely that virulence is an important factor in their selection.

Of all the anaerobes, B. fragilis is the most frequently encountered in intra-abdominal sepsis or bacteremia. Members of the genus Bacteroides were second only to Escherichia coli as a cause of gram-negative septicemia in patients at the Mayo Clinic (Rochester, Minn.), and 78% of these Bacteroides as B. fragilis (7). Studies of intraabdominal sepsis and infection of the female genital tract indicated that B. fragilis is the most common cause of bacteremia in these clinical settings (2,7). Much of our work is focused on this common anaerobe.

Organisms classified as B. fragilis were formerly subdivided into six subspecies: fragilis, distasonis, vulgatus, thetaitaomicron, ovatus, and an unspecified group, subspecies "other." These subspecies share many phenotypic characteristics, including resistance to penicillins, and their separation was based on minor variations in biochemical reactions. Although they have been reclassified into distinct species on the basis of studies of DNA homology (8), the older, more familiar classification is used in this presentation.

The distribution of the B. fragilis subspecies is markedly different in normal flora and infected sites. In the colon, the usual source of B. fragilis in septic processes, the numerically dominant subspecies are distasonis, vulgatus, and thetaitaomicron; subspecies fragilis accounts for only about 0.5% of the colonic microflora (9). In clinical specimens, however, subspecies fragilis is most often encountered. During a two-year period we observed 338 strains of B. fragilis in blood cultures or exudates from clinical infections. Of these organisms, 260 (78%) were subspecies fragilis. Thus, when compared to the numerical concentrations in normal flora, B. fragilis subspecies fragilis is present in a disproportionately large number of clinical isolates (10,11) its predominance in exudate and blood strongly suggests that this subspecies has unique virulence properties.



### The Outer Membrane of *B. fragilis*

We conducted several studies to determine whether any unique bacterial components of subspecies *fragilis* could account for its enhanced virulence. We began with immunochemical analysis of the outer membrane structure, and this investigation provided some insight into the virulence of the components of the membrane.

In *B. fragilis* and other gram-negative bacteria, the cell wall consists of an outer membrane, an inner, or cytoplasmic membrane, and a rigid peptidoglycan layer, which separates the two trilaminar membranes (figure 1).

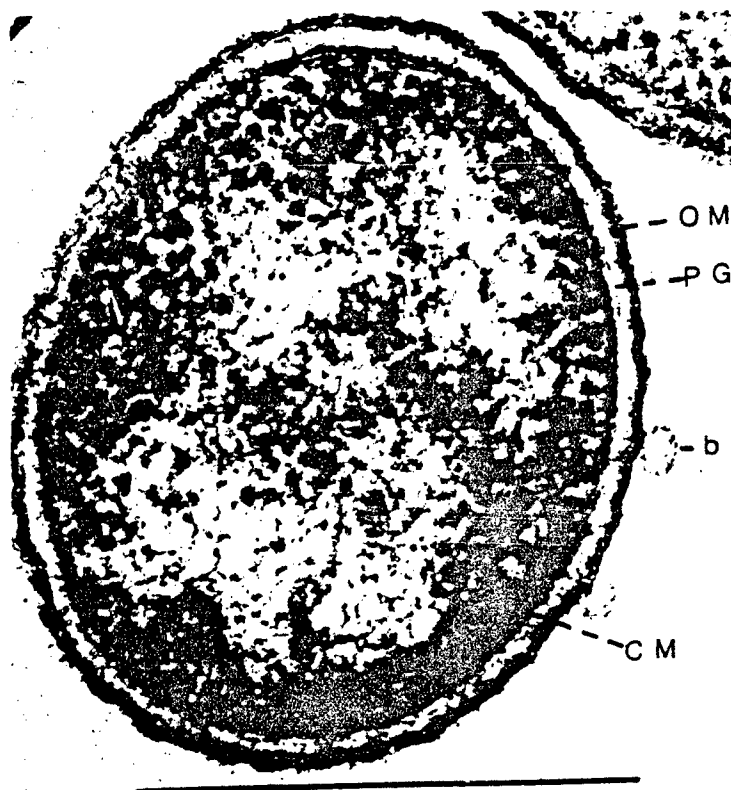


Figure 1. Electron micrograph of *Bacteroides fragilis* subspecies *fragilis* stained by standard techniques ( $\times 120,000$ ). OM = outer membrane; PG = peptidoglycan; b = bleb of outer membrane that is extruded into the growth medium; CM = cytoplasmic membrane. The scale marker denotes 1  $\mu$ m.

The cytoplasmic membrane is generally the site of membrane synthesis and thus contains most of the biosynthetic enzymes. The outer membrane contains the antigens that are encountered by the host, in other words, the surface components of the bacteria.

The outer membranes were loosened from intact *B. fragilis* by heating of organisms that were suspended in a buffer that contained 10 mM EDTA. This procedure was followed by gentle shearing through a 25-gauge needle and purification by differential ultracentrifugation (12). The purity of the membrane fraction was assessed by isopycnic ultracentrifugation on a 30%-65% sucrose density gradient. A single peak was identified (figure 2); its density was 1.23 g/ml, a value similar to that reported for other bacterial outer membranes (13).

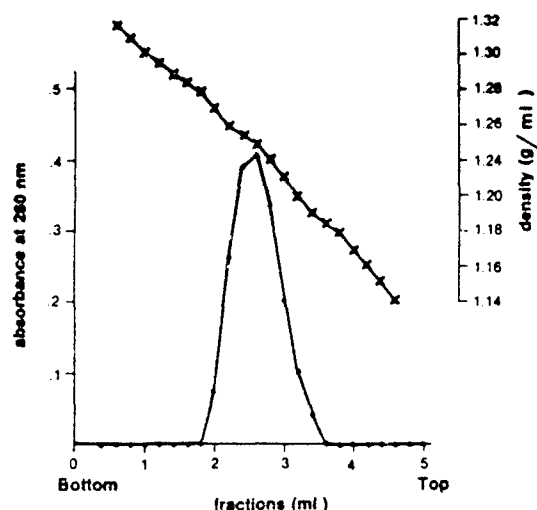


Figure 2. Absorption at 280 nm of fractions collected from a 30% -65% discontinuous sucrose density gradient centrifugation of the outer membrane fraction of *Bacteroides fragilis* subspecies *fragilis* (strain ATCC 23745, American Type Culture Collection, Rockville, Md.). Density (x — x) was determined with a refractometer.

The outer membrane in gram-negative bacteria contains protein, LPS, and loosely bound lipids. Capsular polysaccharide is also present in some bacteria, including *B. fragilis* (14). When we studied the protein component of the outer membranes of strains of *B. fragilis* by means of electrophoresis in sodium dodecyl sulfate-polyacrylamide gels, the representative strains from each subspecies had distinct peptide-band patterns (figure 3, left). This finding was not surprising since the outer membrane proteins in other bacteria are biochemically and genetically heterogeneous. Of greater interest was the observation that strains and subspecies *fragilis* had nearly identical peptide-band patterns (figure 3, right), a similarity that is unusual within a biochemical group.

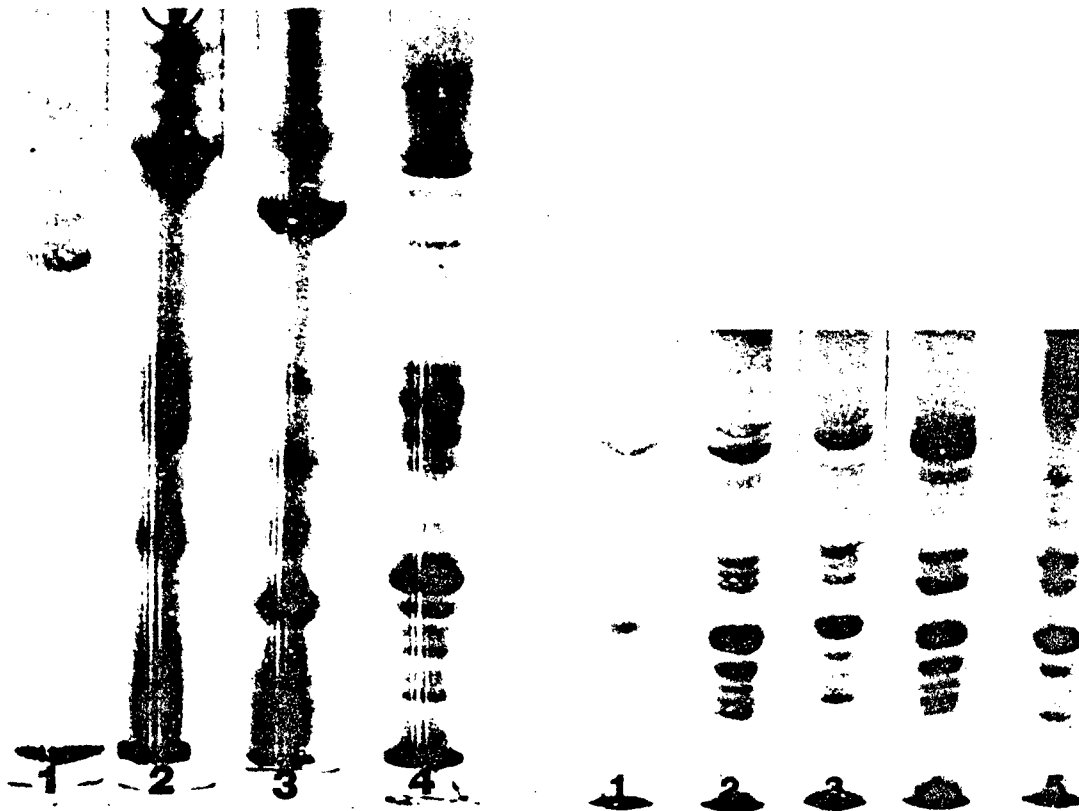


Figure 3. Electrophoretic studies of structural proteins in outer membranes of cells that represent four subspecies of *Bacteroides fragilis* (left) and of cells that represent five strains of *B. fragilis* subspecies *fragilis* (right). Electrophoresis was performed in sodium dodecyl sulfate-polyacrylamide gels. The subspecies shown in the figure on the left are *distasonis* (strain 8503), *vulgatus* (strain 8482), *thetaiotaomicron* (strain 12290), and *fragilis* (strain 23745). The strains of subspecies *fragilis* shown in the figure on the right are 2429 (Los Angeles, Calif.), ATCC 23745 (American Type Culture Collection, Rockville, Md.), 1262 (Philadelphia, Pa.), 2244 (Los Angeles, Calif.), and 26747 (Boston, Mass.). These strains have nearly identical peptide-band patterns.

As stated above, the LPS component is regarded as a major virulence factor of gram-negative bacteria. However, the LPS of *B. fragilis* is biologically distinct from that of aerobic gram-negative bacteria (15). Although, chemically, it is a lipopolysaccharide, it does not function as an endotoxin. The LPS of aerobic gram-negative bacteria contains a lipid (lipid A) portion (figure 4), which consists primarily of a disaccharide backbone highly substituted with long-chain fatty acids (16).

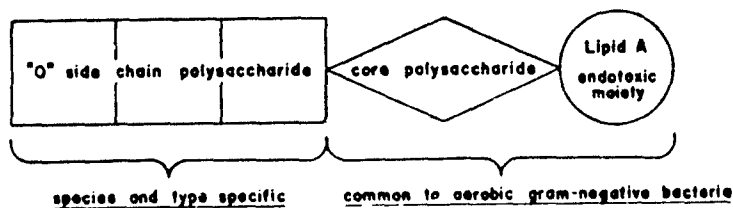


Figure 4. Schematic diagram of the typical lipopolysaccharide or endotoxin of gram-negative bacteria.

This portion is linked to a carbohydrate core, which contains two unusual sugars (2-keto-3-deoxyoctonate and a heptose), as well as glucose, galactose, and glucosamine. The core is linked to the repeating carbohydrates of the O side chain. Most aerobic gram-negative bacteria have biologically similar lipopolysaccharide cores, and antibodies to these cores are cross-reactive between bacterial species. However, the O side chain differs markedly between strains even of the same species and thus is the basis of serologic typing schemes for the Enterobacteriaceae (16). The core of the major pathogenic *Bacteroides* species (*fragilis* and *melaninogenicus*) is quite distinct from the core of other facultative and of at least some anaerobic bacteria (15,17-19).

The lipopolysaccharide of *B. fragilis* contains no 2-keto-3-deoxyoctonate or heptose in the inner core and no  $\beta$ -hydroxy myristic acid, a common constituent of aerobic lipopolysaccharides, in the lipid moiety. Over 98% of the lipid moiety is easily removed by extraction with chloroform-methanol and ethanol, a fact indicating that the lipid is loosely associated with the polysaccharide. Physicochemical studies showed that the LPS of *B. fragilis* has some characteristics of the LPS of other bacteria. The *B. fragilis* LPS can disaggregate to a monomeric unit in the presence of detergents such as sodium deoxycholate, and it reaggregates in the absence of these detergents (15). In biologic studies, it causes gelation of the limulus lysate only at a much higher concentration than that of the endotoxin of *Salmonella typhi*. Furthermore, the LPS of *B. fragilis* is not lethal in 10-day-old chick embryos at doses 100 times higher than the lethal dose of *S. typhi*. Whereas the *S. typhi* LPS induces the local Shwartzman reaction at a dose of 3  $\mu$ g per rabbit; the *B. fragilis* LPS fails to provoke this reaction at a dose of 1 mg per rabbit; furthermore, intact, oxygen-killed *B. fragilis* is ineffective at a dose of 2.5 mg. These findings indicate that the LPS of *B. fragilis* is different from that of most gram-negative bacteria. Although it has some of the chemical and physical properties of an endotoxin, it lacks the biologic properties of a true endotoxin.

An understanding of the LPS component of the *Bacteroides* may have important clinical implications. Patients with bacteremia due to *Bacteroides* rarely have either disseminated intravascular coagulation or purpuric skin lesions, disorders that are clearly attributable to endotoxins, and the absence of these disorders probably reflects the biologic impotence of these LPS.

#### Bacterial Capsules

Another possible virulence factor, the bacterial capsule, is exemplified by the polysaccharide of *S. pneumoniae*. Some strains of *B. fragilis* possess a capsular polysaccharide of large molecular size. Immunochemical purification can be achieved by gentle separation of the polysaccharide from the outer membrane (14). Capsules can be demonstrated by several techniques, and a possible explanation for the higher rates of recovery associated with subspecies *fragilis* as compared with other subspecies found in clinical specimens may be apparent from these studies.

Thirty strains of *B. fragilis*, characteristic of the various subspecies, were studied with the electron microscope and stained with ruthenium red. Fifteen strains of subspecies *fragilis* were examined, and a distinct capsule could be seen on the external surface of the outer membrane of 14 of the 15 strains. Capsules were absent from most strains from the other subspecies; however, with one strain of subspecies *vulgatus*, a dense capsular material was present in 20% of the organisms in the microscopic field. Also, a distinct capsule could be seen in one strain of subspecies *thetaiotaomicron*. In several strains of subspecies other than *fragilis*, a less dense material giving a "hairy" surface appearance to the outer membrane was seen, but it was easily differentiated from the electron-dense capsules of the strains of subspecies *fragilis*. Figure 5 shows eight strains of various subspecies of *B. fragilis*.

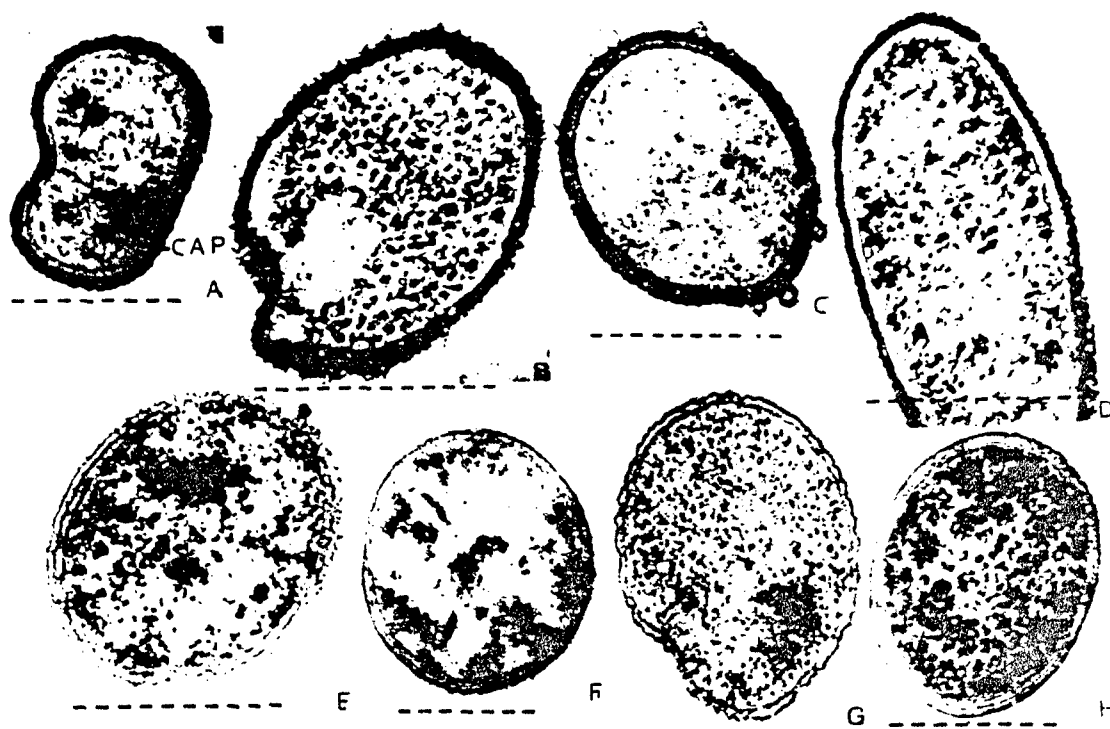


Figure 5. Electron micrographs of cells of four strains of *Bacteroides fragilis* subspecies *fragilis* (top row) and of four strains of the other subspecies of *B. fragilis* (bottom row) stained with ruthenium red for the detection of capsules (CAP) ( $\times 53,800$ ).

Quellung reactions with antiserum specific for the polysaccharide of subspecies *fragilis* demonstrated capsular swelling in all 10 strains of this subspecies. Five strains from each of the other subspecies failed to swell with this antiserum or with antisera to isolates of homologous subspecies. An indirect immunofluorescence assay was developed for identification of clinical isolates; capsular material immunologically similar to that in a reference strain of subspecies *fragilis* was used. In this assay all 132 strains of subspecies *fragilis* that we have studied were positive for capsular material, whereas all but one of 62 strains from the other subspecies were negative (20). The fluorescence-positive strain of other subspecies of *B. fragilis* was of the subspecies *B. thetaiotaomicron*.

The role of encapsulation as a virulence factor in an animal model of intra-abdominal sepsis. Initial experiments for development of an animal model were designed to simulate the septic consequence of colonic perforation in human beings (21,22). Gelatin capsules containing an inoculum that included anaerobes (*Eubacterium*, *Clostridium*, *Bacteroides*, *Peptococcus*, and *Fusobacterium*) and aerobes (*E. coli*, enterococcus) in a ratio of 100:1 (anaerobes:aerobes) were implanted in the peritoneal cavity of Wistar rats. The natural course of infection was biphasic. The early phase (less than five days) was characterized by acute peritonitis with free-flowing exudate; the mortality was 43%. In the second phase all animals that survived for seven days after challenge had intraabdominal abscesses. Quantitative bacteriologic studies of both phases of illness showed an average of 6.2 bacterial species per specimen. The four most prevalent organisms were *E. coli*, enterococci, *Bacteroides*, and *Fusobacterium*. The two aerobes outnumbered the anaerobes in the peritoneal exudates, whereas anaerobes predominated in the abscess contents. Blood cultures were predictably positive for *E. coli* during the early (peritonitis) phase and were seldom positive for this microbe in cultures obtained after the third postoperative day. The use of antimicrobial probes further clarified the role of various organisms in the disease (23). Treatment with gentamicin,

which is inactive against the anaerobes, reduced the early mortality to 4% but failed to prevent abscesses. In contrast, clindamycin, which is inactive against E. coli, failed to reduce early mortality but nearly eliminated formation of abscesses among survivors. A combination of the two antibiotics showed the salutary effects of each in that there was a reduction of both early mortality and the second-phase abscess formation noted in untreated animals. Thus, it appeared that the coliforms were responsible for early death, whereas the anaerobes appeared to cause abscess formation.

In another series of experiments, the animals were challenged with various bacterial species instead of with the complex fecal inoculum. These studies showed that E. coli was required to produce mortality, whereas abscesses occurred only when an anaerobe and an aerobe were combined (24). However, the strain of B. fragilis used in this experiment was subsequently shown to be unencapsulated, and further studies showed that the aerobe was unnecessary as an adjunct for abscess development when encapsulated B. fragilis was used as the anaerobe for infection of animals (25). Subsequently, it was shown that heat-killed B. fragilis produced abscesses indistinguishable from those resulting from infection with viable organisms. Finally, when purified capsular material from B. fragilis was implanted, abscesses again resulted. Control studies including the implantation of capsular polysaccharide of E. coli and heat-killed type III S. pneumoniae, failed to produce detectable disease.

This group of experiments has provided an approach to the study of pathogenesis of anaerobic infection by analysis of specific virulence in humans.

Antibody response to the B. fragilis capsule in the rat peritonitis model. The humoral antibody response to the capsular polysaccharide of B. fragilis was quantitated in animals with intraabdominal abscesses by means of a sensitive radio-active antigen-binding assay (26). The antibody detected by this technique correlated highly with that measured by quantitative precipitin analysis ( $r=0.943$ ). Animals infected with encapsulated B. fragilis had high levels of circulating serum antibody to the capsular polysaccharide (figure 6).

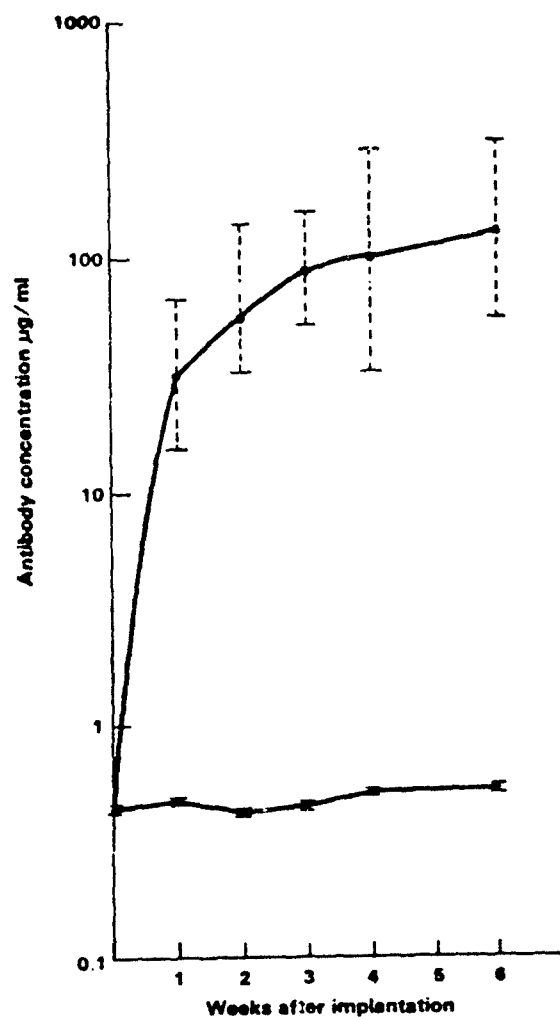


Figure 6. Quantitative antibody response to the capsular polysaccharide of *Bacteroides fragilis* for groups of 10 rats implanted with encapsulated *B. fragilis* (line with dashed brackets) and unencapsulated *B. fragilis* (line with solid brackets). Means  $\pm$  so are plotted for each weekly sampling of blood after implantation (week zero). Implants for both groups included  $5 \times 10^7$  viable enterococci, sterile cecal contents,  $\text{BaSO}_4$ , and either the encapsulated *B. fragilis* or the unencapsulated strain of *Bacteroides*.

This antibody could be induced by implantation of live organisms, heat-killed organisms, heterologous strains of *B. fragilis*, or various outer-membrane components that contained the capsular antigen, and the immunogenicity of the capsular polysaccharide could be enhanced by complexing to the outer membrane or to outer-membrane proteins.

Antibody response of women with pelvic inflammatory disease to the capsular polysaccharide of *B. fragilis*. By use of the radioactive antigen-binding assay, the antibody response to the *B. fragilis* capsule was quantitated in women with acute pelvic inflammatory disease (27). Table 1 lists the antibody levels in paired sera from 33 patients, the interval (in days) between collection of the two samples of serum, and the change in antibody concentration during the interval.

Table 1. Results of culture and concentrations of serum antibody to the capsule of *Bacteroides fragilis* subspecies *fragilis* in 33 selected cases of pelvic inflammatory disease (PID). (Reprinted from *The Journal of Infectious Diseases* [28] with permission of the publisher.)

Group, patient no.	PID episode*	Neisseria gonorrhoeae isolated from cervix	Cul-de-sac isolate(s)		Antibody concentration in μg/ml (interval in days)		Change in antibody concentration (μg/ml)†
			B. fragilis subspecies	Other organism	Acute-phase serum	Convalescent- phase serum	
Group 1							
201	I	-	fragilis, thetaiotaomicron	Aerobes	9.86 (2)‡	14.26 (7)§	4.40
219	I	-	fragilis	Aerobes, anaerobes	31.79 (7)	19.08 (30)	12.71
247	R	-	fragilis	Anaerobes, aerobes	16.75 (1)	9.87 (14)	6.88
263	I	+	fragilis, distasonis	Anaerobes, aerobes	12.89 (1)	11.45 (34)	1.44
264	I	-	fragilis	Aerobes	30.55 (1)	59.69 (5)	29.14
282	I	-	fragilis	Aerobe	24.26 (2)	10.66 (14)	13.60
300	I	-	fragilis	Anaerobes, aerobe	12.78 (6)	17.89 (20)	5.11
259	R	-	Unavailable	Bacteroides capillosus, anaerobes, aerobe	16.39 (14)	25.21 (23)	8.82
069	R	-	Unavailable	...	14.74 (1)	22.64 (7)	7.90
Group 2							
233	I	-	distasonis	Anaerobes, aerobes	23.13 (10)	21.65 (46)	1.48
245	I	-	distasonis, vulgatus, thetaiotaomicron	Anaerobes, aerobe	15.15 (14)	13.33 (21)	1.82
268	I	-	...	Anaerobes	12.03 (7)	13.16 (21)	1.13
301	I	-	...	Anaerobes	13.66 (15)	11.65 (10)	2.01
248	R	-	...	Anaerobe, aerobes	45.55 (1)	43.05 (10)	2.50
258	R	-	...	Anaerobe, aerobe	20.78 (7)	17.10 (28)	3.68
262	I	-	...	Aerobes, Mycobacterium hominis	9.44 (4)	15.49 (14)	6.05
202	R	-	...	Aerobe	15.14 (1)	12.73 (34)	2.41
209	I	-	...	Aerobe	12.37 (1)	13.65 (180)	1.28
229	I	-	...	Aerobes	17.36 (1)	18.77 (33)	1.41
243	I	-	...	Aerobe	8.01 (1)	8.51 (34)	0.50
269	I	-	...	Aerobe	8.63 (5)	8.73 (21)	0.10
272	I	-	...	Aerobe	14.24 (4)	24.60 (18)	10.36
290	R	-	...	Aerobe	9.88 (4)	8.57 (14)	1.31
Group 3							
206	I	+	distasonis	Aerobes	8.18 (2)	9.69 (20)	1.51
212	R	+	...	N. gonorrhoeae, B. capillosus, aerobe	13.51 (2)	13.45 (30)	0.06
235	I	+	...	N. gonorrhoeae, aerobe	19.47 (10)	19.84 (64)	0.37
276	I	+	...	N. gonorrhoeae	22.21 (14)	11.21 (14)	10.99
284	I	+	...	N. gonorrhoeae	22.59 (2)	20.28 (20)	2.21
295	I	+	...	N. gonorrhoeae, aerobe	14.28 (1)	14.84 (34)	0.56
307	I	+	...	N. gonorrhoeae	28.88 (1)	26.67 (6)	2.21
308	R	+	...	N. gonorrhoeae	26.49 (3)	17.70 (9)	8.79
227	I	+	No growth		15.02 (2)	12.87 (60)	2.15
236	I	+	No growth		11.66 (3)	10.40 (18)	1.26

NOTE. Tables with total identification of all isolates are available upon request from the authors.

\*I = initial, R = recurrent.

†The mean changes for groups 1, 2, and 3 were 10.00, 2.58, and 3.01, respectively.

‡Interval from onset of abdominal pain until sample was obtained. I = indeterminate.

§Interval between samples.



Patients were categorized according to the results of cultures of fluid from their cul-de-sac and cervical swabs; those yielding B. fragilis subspecies fragilis alone or in mixed cultures from the cul-de-sac are listed first (group 1), followed by those yielding strains of Bacteroides that were not subspecies fragilis (group 2), and those with no evidence (on the basis of culdocentesis cultures) of infection with Bacteroides (group 3). The mean difference in concentrations of serum antibody between acute- and convalescent-phase specimens for women with B. fragilis subspecies fragilis was 10.47  $\mu\text{g/ml}$  (group 1). For those with cervical cultures negative for Neisseria gonorrhoeae, the mean change was 2.58  $\mu\text{g/ml}$  (group 2). The mean change in antibody concentration in the first group was significantly higher than for each of the other groups or for a group of healthy female volunteers ( $P < 0.01$ , one-way analysis of variance). Changes in antibody levels in group 2 were not significantly different from those of volunteer laboratory workers. These results demonstrate much greater changes in levels of serum antibody to the capsule of B. fragilis subspecies fragilis in patients from whom this organism was isolated by culdocentesis or laparoscopy as compared with changes in antibody levels of other patients.

The data support the bacteriologic evidence (28) that B. fragilis subspecies fragilis is a more important etiologic agent in acute nongonococcal than in gonococcal pelvic inflammatory disease; however, these rises in titer of antibody cannot be interpreted as absolute proof of causality of the infection. This argument could be misleading; one needs only to be reminded of the frequent finding of Neisseria meningitidis in the nasopharynx of military recruits with pharyngitis due to adenovirus disease. These recruits simultaneously develop antibodies to N. meningitidis and to adenovirus; yet N. meningitidis is not the primary pathogen. The clinical setting of pelvic inflammatory disease and B. fragilis is not completely analogous, however, B. fragilis is a part of the normal colonic flora of humans. Therefore, the isolation of this organism from an infected site with a concomitant rise in titer of antibody, although not fulfilling Koch's postulates, does provide supportive evidence for the causation of disease.

Antibody response of women with abscesses after hysterectomy of the capsular polysaccharide. To determine the possible role of B. fragilis in the development of infectious complications of hysterectomy, we measured serum antibody to the capsular polysaccharide in 53 women before and several days after elective surgery (29).

Specimens of serum were collected from potential participants before surgery. At the time of discharge, a second specimen of serum was collected from five categories of patients: (1) those with an uncomplicated postoperative course; (2) those with febrile illness (oral temperature of  $\geq 100.4$  F on at least two days after surgery, excluding the first 24 hr); (3) those with abdominal or wound infection; (4) those with pelvic cellulitis; and (5) those with abscess (cuff abscess or pelvic abscess).

The base-line concentrations of antibody of these individual subjects varied widely (2.0-17.7  $\mu\text{g/ml}$ ), as did the final concentrations (table 2).

ons of concentration of  
of Bacteroides fragilis  
dergoing hysterectomy.

Median antibody concentration in $\mu\text{g/ml}$ (range)	
Before surgery	After surgery *
5.2 (2.0-11.3)	6.6 (2.5-10.6)
4.1 (3.1-4.8)	5.5 (3.6-8.7)
4.3 (3.7-10.3)	5.5 (3.6-10.4)
5.6 (3.1-17.7)	13.0 (4.0-17.9)
5.6 (3.0-13.3)	7.8 (3.2-13.0)

\* Concentration was measured in samples of sera  
at time of discharge from the hospital.

The changes in antibody concentrations for each of the five groups are listed by diagnosis in table 2. Analysis of variance demonstrated that the five groups differed significantly in their mean changes in concentration of antibody between the pre- and postoperative specimens ( $F_{4,48}=6.54$ ;  $P < 0.05$ ). Further analysis revealed that the mean rise in concentration of antibody ( $4.91 \mu\text{g/ml}$ ) in the group with abscesses was significantly greater than the mean rise among patients with no complications ( $0.62 \mu\text{g/ml}$ ), wound infection ( $1.90 \mu\text{g/ml}$ ), pelvic cellulitis ( $0.63 \mu\text{g/ml}$ ), and febrile illness ( $0.95 \mu\text{g/ml}$ ) ( $t=4.52$ ;  $P < 0.05$ ). The mean changes in antibody concentrations among the latter four groups of patients were not significantly different from each other ( $t=1.35$ ;  $0.20 < P < 0.10$ ). Two of the five patients without abscess who had an increase in antibody concentration of  $>3.0 \mu\text{g/ml}$  had pathologic findings of pelvic inflammatory disease. Another of these five patients had a wound infection from which were cultured two types of anaerobic gram-negative bacilli that were not identified further.

The data presented here suggest that the majority of abscesses following hysterectomy, either cuff or pelvic abscesses, are associated with an increase in antibody to the capsular polysaccharide of B. fragilis. The data further suggest that, with the possible exception of wound infections, the other infectious or presumably infectious complications of this procedure are uncommonly associated with such changes in antibody concentrations.

#### Protective Efficacy of Immunization with Capsular Antigen Against Experimental B. fragilis infection.

The protective efficacy afforded by immunization with the capsular antigen against abscess formation and bacteremia with Bacteroides fragilis was studied in an experimental rat model of intra-abdominal sepsis (30).

Over 90% of non-immunized animals; of animals immunized with methylated bovine serum albumin and complete Freund's adjuvant, or animals immunized with B. thetaiotaomicron lipopolysaccharide developed abscesses when challenged intraperitoneally with either of 2 strains of B. fragilis given with or without an enterococcus, or B. distasonis with an enterococcus or the cecal contents of meat fed rats.

Animals immunized with capsular polysaccharide, given with or without methylated bovine serum albumin and complete Freund's adjuvant, or animals immunized with the outer membrane of one B. fragilis strain (23745) were significantly protected from abscesses caused by challenge with Bacteroides fragilis or Bacteroides distasonis. Immunization had no overall effect on the development of abscesses in animals challenged with the entire cecal contents, however B. fragilis was eliminated from the abscesses of these animals.

Animals immunized with the capsular polysaccharide were protected from early bacteremia with B. fragilis.

The evidence summarized here that (1) B. fragilis is very common in infections of humans where anaerobes are likely to be found, (2) that B. fragilis is clearly involved in the pathogenesis of experimental intraperitoneal abscesses, (3) that humans make antibody to the capsular polysaccharide of B. fragilis in natural infection and (4) that experimental infection with B. fragilis can be prevented by immunization with the capsular polysaccharide; all indicate that prevention of this disease by immunization is a feasible approach.

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Section II

Rapid diagnosis of Bacteroides infection by  
indirect immunofluorescence of clinical specimens.

## Introduction

The Bacteroides fragilis group of bacteria consist of a number of species which are the anaerobic bacteria most frequently isolated from clinical infections. (1,2) Amongst these species, Bacteroides fragilis is the most important, accounting for over 70% of the strains isolated, (3) despite its low prevalence in the normal gastrointestinal flora. (4,5) B. fragilis possess pathogenic properties which have not been demonstrated in other species. (6-8) It has been shown that a capsular polysaccharide unique to B. fragilis, represents a virulence factor. (9)

Antiserum to this purified capsular polysaccharide was raised in rabbits. (10) An indirect immunofluorescence assay (I.F.A.) was used to demonstrate that this capsular antiserum was specific against B. fragilis, and could be used to differentiate this species from other species of the Bacteroides fragilis group (B. thetaiotaomicron, B. vulgatus, B. distasonis, B. ovatus, other Bacteroides species). (10)

A number of serogroups of the Bacteroides spp. based on O-antigens have been described. (11-13) Type-specific antisera raised in rabbits against twenty distinct serotypes were used to serotype clinical strains of Bacteroides species. A pool of antisera against the most predominant serotypes has been shown in an I.F.A. to identify Bacteroides sp. (unpublished data).

The diagnosis of the Bacteroides fragilis group of organisms in infections is important because of the specific antibiotic therapy required. The routine bacteriological techniques are time-consuming and we therefore investigated the possible use of the capsular and the pooled antisera in an I.F.A. applied directly to clinical specimens. The aim is to identify Bacteroides sp. in clinical material and provide a rapid diagnosis.

## Methods

### Specimens

A total of 43 clinical samples were examined by an I.F.A. and by routine bacteriological methods. The type of specimen, numbers, and means of transport are shown in table I.

TABLE I—SUMMARY OF RESULTS OBTAINED BY I.F.A. AND BACTERIOLOGICAL METHODS

Specimen	No.	Transport method	Number positive in:		
			Capsular antiserum I.F.A.	Pooled antiserum I.F.A.	Culture for <u>B. fragilis</u> / <u>Bacteroides</u> sp.
Pus	12	Bottle*	8	10	5/6
Aspirate	4	Bottle*	1	2	0
Pelvic swab	6	Amies medium	2	5	2/2
Pelvic swab	2	Dry swab	0	0	0
Wound swab	3	Amies medium	0	1	0
Wound swab	14	Dry swab	4	5	3/4
Perineal swab	1	Dry swab	0	1	0/1
Pus	1	Plain container	0	1	0
Total	43		15	25	10/13†

\*Gassed out transport bottle.

†6 culture positive specimens yielded two different Bacteroides sp.

Consecutive specimens taken into gassed-out transport bottles were included in the study but some pre-selection of the swabs was made where anaerobic sepsis seemed most likely.

#### Bacteriological Methods

All the specimens were examined by direct microscopy of Gram-stained films, and aerobic and anaerobic cultures were set up. The specimens received in gassed-out bottles were processed in an anaerobic chamber based on the glove-box isolator, (16) and were also analysed by gas-liquid chromatography (G.L.C.) for the presence of short-chain fatty acids (S.C.F.A.). (17) Pre-reduced media were used for primary anaerobic culture and for subsequent identification of anaerobic Gram-negative rods. All Bacteroides were identified to species level using high-content antibiotic disc sensitivities, G.L.C., and the A.P.I. 20 anaerobe system. The pure cultures were then tested by I.F.A. for confirmation.

#### Preparation of Antisera

Purified capsular polysaccharide was prepared from the outer membrane of B. fragilis strain A.T.C.C. 23745. Capsular antiserum to this was raised in rabbits. (9,10)

Whole-cell antisera to cultures of different serotypes of B. fragilis were also raised in rabbits. (14) Cross-reacting antigens were not absorbed out and a pool of antisera was made of equal dilutions in phosphate-buffered saline (P.B.S.).

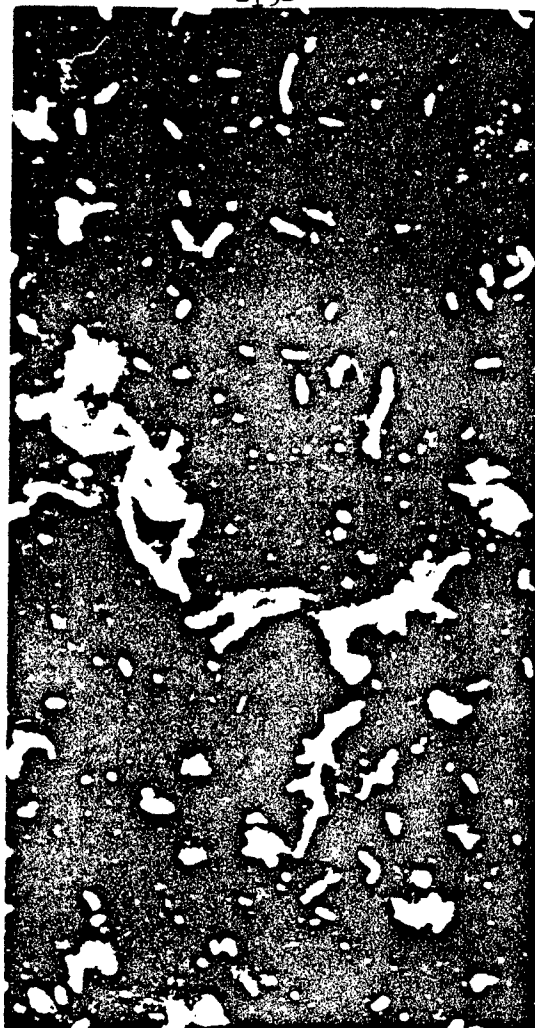
Serum taken from rabbits before inoculation of antigen was used as a control and gave no fluorescence on I.F.A. with any Bacteroides sp.

#### I.F.A.

Thin smears of each specimen were made in the wells of 'Teflon'-coated slides prepared before use as described elsewhere (18). Slides smeared with the clinical material were allowed to dry, then gently heat-fixed. Small drops of capsular and pooled antisera diluted 1/32 in P.B.S. were applied to specified wells and control serum was added to duplicate samples. The slides were incubated for 30 minutes in a moist chamber at room temperature, washed in P.B.S. for 10 minutes, rinsed in distilled water, and dried.

Fluorescein-labelled anti-rabbit immunoglobulin raised in sheep (Wellcome Reagents, Ltd.) and diluted 1:10 in P.B.S. was added to each well in small drops. Again the slides were incubated for 30 minutes in a moist chamber at room temperature, washed in P.B.S. for 10 minutes, rinsed in distilled water, and dried. A large coverslip was mounted using buffered glycerol and the slides were read at a magnification of x40 with a Leitz fluorescence microscope. Incident-light excitation was produced using the lamp housing 100Z with a 50 W ultra-high pressure mercury lamp and the 'Ploemopak 2.2' and K.P. 500 exciting filter in conjunction with the Ortholux II fluorescence microscope. Results were recorded on a scale of 0 to 4+, where 4+ represented bright fluorescence (figure) and 1+ faint fluorescence.





Positive (4+) I.F.A. of a specimen of pus (table II, specimen 11).

Scores of 0, 1+, or 2+ with the antiserum were called fluorescence-negative. Specimens showing 3+ or 4+ with the antiserum, but which were fluorescence-negative with the control serum, were recorded as positive. An attempt to count the number of organisms per field of view was made.

#### Statistical Analysis

The sensitivity (positive I.F.A. test/total positive (culture or G.L.C.)), specificity (negative I.F.A. test/total negative (culture or G.L.C.)), false positives (positive I.F.A. test/total negative (culture or G.L.C.)), and false negatives (negative I.F.A. test/total positive (culture or G.L.C.)) for each test were determined by comparison of the I.F.A. results with either culture, S.C.F.A. examination by G.L.C., or both. (17,19) On samples of pus (table II), where G.L.C. could be done, data analysis was done with the knowledge that S.C.F.A are not species-specific in the B. fragilis group.

TABLE II—RESULTS OF I.F.A. AND BACTERIOLOGICAL ANALYSIS OF 12 SPECIMENS OF PUS

Specimen source	Gram films	I.F.A. with		G.L.C.	Anaerobic culture	Aerobic culture
		Capsular antiserum	Pooled antiserum			
Empyema	N.S.	—	—	—	N.G.	N.G.
Abdominal wound	G.P.C.	—	—	—	N.G.	<i>Staphylococcus aureus</i>
Intra-abdominal abscess	G.N.R.	+	+	+	<i>B. fragilis</i> Anaerobic streptococcus Microaerophilic streptococcus	<i>Staphylococcus aureus</i>
Uterine abscess	G.N.R., G.P.C., G.P.R.	+	+	+	<i>B. fragilis</i> <i>Actinomyces israeli</i> Anaerobic streptococcus	N.G.
Subphrenic abscess	G.N.R., G.P.C., G.P.R.	+	+	+	<i>B. fragilis</i>	Coliform <i>Pseudomonas</i> sp. <i>Candida</i> sp.
Intra-abdominal abscess	G.N.R., G.P.C., G.P.R.	—	+	+	<i>B. melaninogenicus</i> <i>B. distasonis</i>	Coliform <i>Candida</i> sp. <i>Pseudomonas</i> sp.
Vulval abscess	G.P.C.	+	+	+	Microaerophilic streptococcus	
Intra-abdominal abscess	G.N.R., G.P.C.	+	+	+	N.G.	<i>Proteus</i> sp. Coliform
Intra-abdominal abscess	G.N.R.	+	+	—	N.G.	<i>Streptococcus faecalis</i> Coliform <i>Proteus</i> sp.
Leg abscess	N.S.	—	+	—	N.G.	N.G.
Intra-abdominal abscess	G.N.R., G.P.C.	+	+	+	<i>B. fragilis</i> <i>B. vulgatus</i> Microaerophilic streptococcus	Coliform
Abdominal wound	G.N.R., G.P.C.	+	+	+	<i>B. fragilis</i> <i>B. vulgatus</i> <i>Clostridium welchii</i>	Coliform <i>Streptococcus faecalis</i>

N.S.: no bacteria seen; G.N.R.: gram-negative rods; G.P.C.: Gram-positive cocci; G.P.R.: Gram-positive rods; N.G.: no significant bacterial growth; G.L.C.: gas liquid chromatography (where + signifies detection of short chain fatty acids).

The predictive value of a positive and negative test was determined by the method of Vecchio. (20)

### Results

43 specimens were examined using both sera in the I.F.A. assay (table I). Table II details the results of analysis of one subgroup of specimens from Table I, those with pus which were received in gassed-out transport bottles.

Using the I.F.A. on clinical specimens, both the anticapsular serum and pooled antisera were extremely sensitive for the detection of *Bacteroides* strains (tables III and IV).

TABLE III—INDIRECT FLUORESCENT ANTIBODY ASSAY USING *B. fragilis* ANTICAPSULAR SERUM

		I.F.A. test	
		Positive	Negative
<i>B. fragilis</i> culture and/or G.L.C.	Positive	12	0
	Negative	3	28

TABLE IV—INDIRECT FLUORESCENT ANTIBODY ASSAY USING *B. fragilis* GROUP POOLED WHOLE ORGANISM ANTISERUM

		I.F.A. test	
		Positive	Negative
<i>Bacteroides</i> group culture and/or G.L.C.	Positive	15	0
	Negative	10	18

The anticapsular serum (table III) detected all twelve B. fragilis strains that were either cultured or identified by S.C.F.A. analysis. The pooled whole organisms sera (table IV) detected all 15 specimens with Bacteroides species (including B. fragilis). Neither I.F.A. test gave any false negative results. The anticapsular serum was very specific for B. fragilis with 28 of 31 cultures and/or G.L.C. negative specimens giving negative results (specificity 90.3%). The pooled whole organism sera I.F.A. showed negative specimens (specificity 64.3%). The false-positive rate was 9.7% for the B. fragilis capsular serum and 35.7% for the pooled serum indicating that the capsular serum I.F.A. was more specific in detecting B. fragilis than was the pooled serum I.F.A. in detecting Bacteroides group organisms in clinical specimens.

The predictive value positive (p.v. pos) is the likelihood that a subject with a positive I.F.A. test will actually have B. fragilis infection (20) and the p.v. pos was 80% for the capsular I.F.A. The predictive value of a negative capsular I.F.A. test (p.v. neg) was 100%, indicating that a negative I.F.A. assay is a strong predictor of the absence of B. fragilis from the specimen. (20) The p.v. pos for the pooled serum I.F.A. test was 60% for the Bacteroides group infection and the p.v. neg was 100%.

### Discussion

Conventional culture methods for the diagnosis of anaerobic infection are slow and often unreliable. Improved techniques in anaerobic bacteriology, such as pre-reduced media and provision of a suitable anaerobic atmosphere, have increased the isolation rate of anaerobic organisms in the laboratory. This, however, has allowed the isolation of several organisms from specimens which previously may have been thought sterile or to contain only a single species, further delaying the transfer to the physician of information which might directly influence therapy.

Gram-stained films are particularly useful for identification of anaerobic Gram-positive rods but do not sufficiently distinguish anaerobic from aerobic Gram-negative bacteria. More rapid methods of identifying anaerobes are now available but have limited application. Gas-liquid chromatography can now be a very valuable adjunct to diagnosis, (17,21) but again, particularly in mixed cultures, differentiation of species is not always possible. G.L.C. is also expensive, requires a good deal of technical expertise, and is only adaptable to specimens which contain pus or fluid.

In this study, a simple immunofluorescence assay is described which can be directly applied to clinical specimens to identify organisms of the Bacteroides fragilis group. Of the various anaerobes these are the most essential to identify clinically because they are the most common infecting agents and also have unusual antibiotic resistance patterns. This test offers the ability to rapidly and reliably identify Bacteroides fragilis. A diagnosis can be provided within 2 hrs. of laboratory receipt of a specimen. Using two antisera, made to either the capsular polysaccharide of B. fragilis or to whole organisms of the Bacteroides fragilis group, no false negative tests were observed, indicating the extreme sensitivity of these assays. The I.F.A. test with the capsular serum was more specific (90.3% specificity) for detecting B. fragilis than was the I.F.A. test with pooled whole organisms antisera for detecting Bacteroides group organisms in clinical specimens (64.3% specificity).

Because of the excellent predictive value of a negative test in both assays when compared to culture and/or G.L.C. results (p.v. neg 100%) a decision on appropriate antimicrobial therapy can be made with assurance that a patient will not be deprived of appropriate therapy. The predictive value of a positive test for B. fragilis using the capsular serum is quite high (p.v. pos 80%) and so using

this test to decide on antibiotic therapy would decrease the number of patients unnecessarily exposed to potentially toxic antimicrobial therapy. The predictive value of a positive test for the other Bacteroides group using the pooled serum is not as high (p.v. pos 60%), but this still offers better information on which to base therapeutic approaches than that given by current techniques. Because no population prevalence information is available on the diseases in a general population, these predictive values are likely to be maximum values (20). In using rapid diagnostic tests it is best to minimize errors by increasing the specificity of the test, even at the cost of decreased sensitivity (20). For this reason it may be advantageous to use both the sera described here for diagnostic purposes, allowing identification of all organisms of the B. fragilis group (B. fragilis, B. thetaiotaomicron, B. vulgatus, B. distasonis, B. ovatus).

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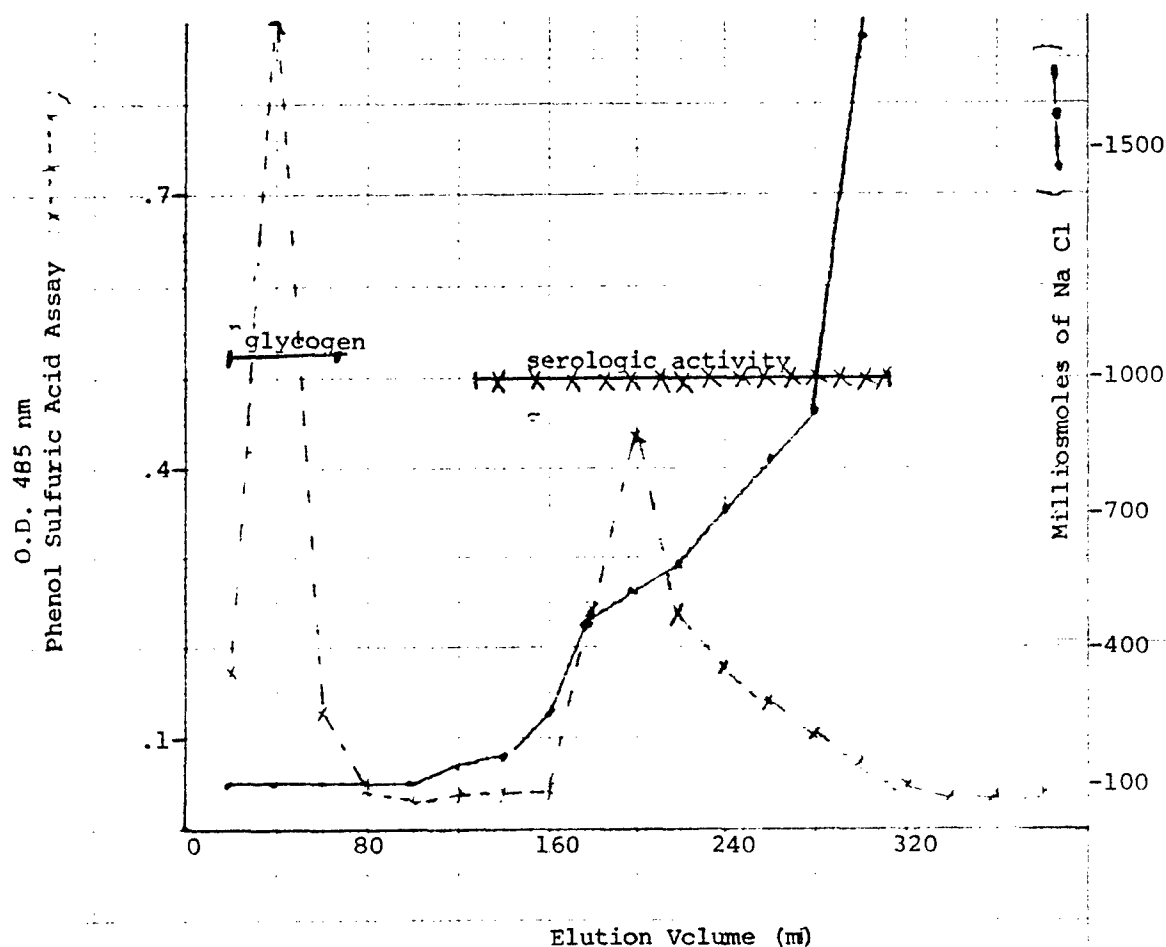
### Section III

Summary of studies on the chemical analysis  
of the capsular polysaccharide and its safety in  
experimental animals.

# I. Evaluation of the Variation in Capsular Structure

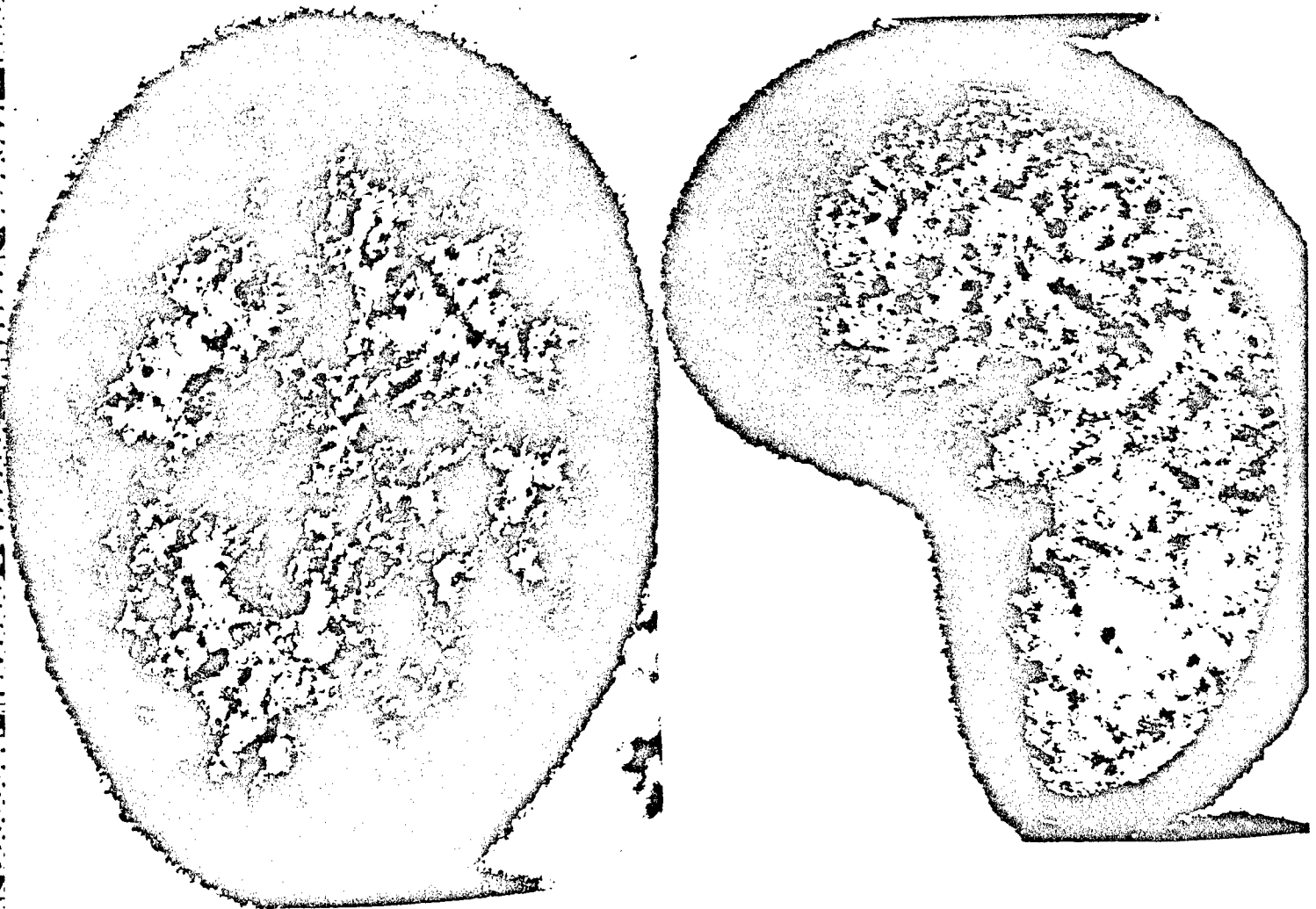
In last years annual report, data was presented demonstrating that in vitro passage of *B. fragilis* induced a variation of capsular structure which had a high glucose content when compared to the low glucose content of the organism with limited passage in vitro. We found that when the "high glucose" capsule producing organism was passed in vivo in the rat abscess model, the capsule reverted to the "low glucose" type. There was no similar change in the LPS or outer membrane proteins.

Studies carried out during this year have shown that the "high glucose" capsule is in fact 2 molecules; the immunologically active capsular polysaccharide and a non-antigenic glycogen which is apparently transported to the cell surface during in vitro passage of the organism. We know the glycogen exists in the cell under non-passed conditions because it is extractable by 10% cold TCA. It is, however, not found in the EDTA extraction of the outer membrane from the surface of these organisms. After several in vitro passages, however, and EDTA extraction, the purified outer membrane complex contains 2 polysaccharides. These are separable by ion exchange chromatography on DEAE sephacel in 0.5M TRIS HCl buffer pH 8.6. (Figure 1)



The glycogen is not retained by the column, while the capsular antigen elutes with an NaCl gradient at 530 milliosmoles. The structure of the glycogen as  $\alpha 1,4$ -1,6 linked glucose has been determined by Drs. Alf Lindberg and J. Lundgren of Stockholm.

Electron microscopic studies were done to define the structural changes in the capsule which take place with passage. In the organisms with normal capsular material a very dense layer can be seen surrounding the cells if staining is done with ruthenium red. A less dense area around the cells can be seen in the cells from which glycogen is isolated from the surface structure. (Figure 2)

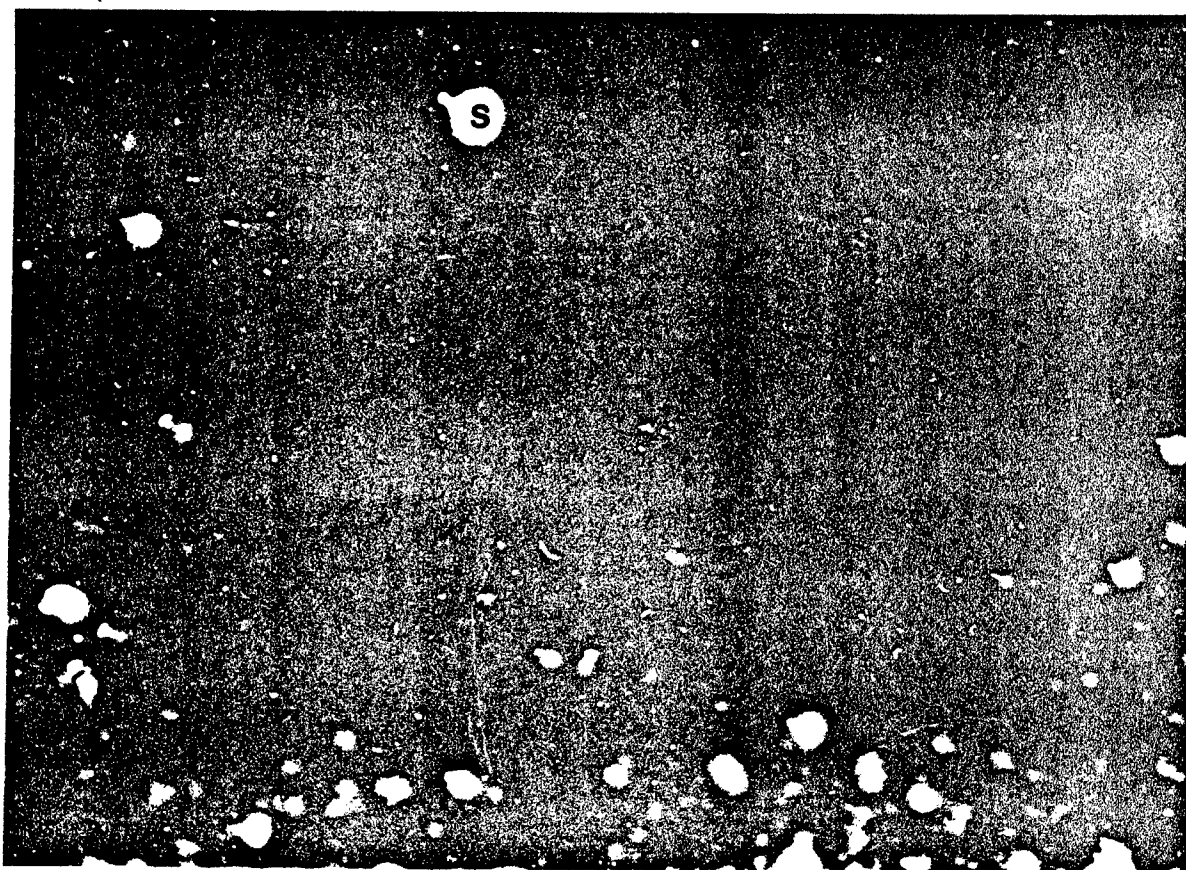


Electron micrograph of B. fragilis strain 23745 after 11 in vitro passages, stained with Ruthenium red.

Non passed strain 23745, similarly stained. Note dense capsule of organism on the right.

It needs to be reemphasized that there is still normal capsular material present on these cells, it is just not as abundant. These cells are however still fluorescent positive in the IFA assay (1) for capsular polysaccharide. There are two colony types of B. fragilis associated with this capsular variation. Figure 3, the smooth normal capsule producing strain is compared to the rough glycogen producing variant.





Smooth(S) and Rough(R) colonial variation of B. fragilis which occurs with in vitro passage. All surface glycogen is associated with the Rough colony types.

These differences have been confirmed repeatedly by isolation of antigen from each colony type. Passage of the rough appearing colony through the rat peritoneal abscess mold reverts the colonial morphology to smooth.

#### Further Purification and Chemical Analysis of Capsular Antigen

A great deal of effort this year has gone into the chemical analysis of the B. fragilis capsule. Unfortunately, I can't report a great deal of progress at this point. There is a discrepancy in content between two methods of carbohydrate analysis. By trimethyl-silylation we can separate several peaks on GLC which we have not been able to identify. They are all constituents with retention times shorter than galactose. Each batch has 10-30% glucose, galactose and glucosamine, but the remainder of the peaks will be identifiable only with the help of mass spectroscopy. We therefore have enlisted the aide of Drs. Alf Lindberg and J. Lundgren in Stockholm in structural analysis of the antigen. Using alditol acetate derivatives, they have not been able to define these early GLC peaks. Amino acid analysis and 260 absorption indicate the purity of the preparation with <1% proteins or nucleic acid present. The inability to convert these sugars to alditols indicates that there may be a group on C-1 which is not an aldehyde.

This group may be a ketose or a carboxyl group. We had thought this end group may have been phosphorylated but we were not able to detect phosphorous by the Bartlett method. The possibility must be considered that these early peaks are lipids, however, gentle hydrolysis followed by hexane or chloroform-methanol extraction does not remove these constituents. The antigen is negatively charged, readily binds to DEAE-sephacel and elutes at a relatively high molarity. This indicates that we may have a fairly large content of acidic sugar or sugars.

### Steps Toward Vaccine Development

#### Toxicity Studies

##### 1. Mouse toxicity tests

Twenty-one CD-1 female mice weighing between 14-16 g were injected intraperitoneally with either 0.5 ml sterile saline (11 mice) or 50 $\mu$ g *B. fragilis* capsule in 0.5 ml saline (10 mice). The mice were weighed daily for 13 days and observed for systemic toxicity or failure to gain weight. No toxicity was observed in any mouse.

mean weight (grams  $\pm$  S.D.)

Day	1	2	3	4	5	6	7	8	9
Control	16.8 $\pm$ 0.3	17.9 $\pm$ 1.0	18.3 $\pm$ 1.3	19.1 $\pm$ 1.2	19.5 $\pm$ 1.7	19.6 $\pm$ 1.3	19.9 $\pm$ 1.4	20.2 $\pm$ 1.5	20.8 $\pm$ 1
Vaccine	16.8 $\pm$ 1.0	17.3 $\pm$ 1.0	17.9 $\pm$ 1.4	18.7 $\pm$ 1.4	19.5 $\pm$ 1.5	18.9 $\pm$ 1.5	19.3 $\pm$ 1.5	19.8 $\pm$ 1.5	20.4 $\pm$ 1
Day	10	11	12	13					
Control	21.0 $\pm$ 1.4	21.6 $\pm$ 1.4	21.8 $\pm$ 1.4	23.0 $\pm$ 1.5					
Vaccine	20.7 $\pm$ 1.5	20.9 $\pm$ 1.0	21.0 $\pm$ 1.2	22.4 $\pm$ 1.3					

##### 2. Guinea Pig Toxicity Tests

Hartley Strain guinea pigs were immunized with either 5 ml of PBS (3 pigs) or 500 g of *B. fragilis* capsular antigen in 5 ml PBS (7 pigs). The animals were weighed and observed daily for 13 days. All vaccine injected guinea pigs continued to grow at the same rate as PBS injected pigs. No toxicity was observed.

mean weight (grams  $\pm$  S.D.)

Day	1	2	3	4	5	6	7	8	9	10	11
Control	278 $\pm$ 15	303 $\pm$ 22	296 $\pm$ 23	285 $\pm$ 20	327 $\pm$ 16	322 $\pm$ 21	326 $\pm$ 22	306 $\pm$ 17	348 $\pm$ 26	352 $\pm$ 22	365 $\pm$ 27
Vaccine	281 $\pm$ 22	305 $\pm$ 19	292 $\pm$ 24	280 $\pm$ 20	323 $\pm$ 23	331 $\pm$ 25	332 $\pm$ 26	315 $\pm$ 26	353 $\pm$ 31	366 $\pm$ 33	376 $\pm$ 37
Day	12	13									
Control	379 $\pm$ 31	393 $\pm$ 37									
Vaccine	374 $\pm$ 30	399 $\pm$ 34									

Based on these safety tests it is reasonable to plan the phase and experiments.

Reference

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#### Section IV

##### Experimental Studies in animal models

- A. Inactivation of chloramphenicol by obligate anaerobes
- B. Deduction of mortality by metronidazole
- C. Development of a model simulating wound abscess

A. Inactivation of chloramphenicol by obligate anaerobes. The administration of chloramphenicol to 60 rats implanted with cecal inocula resulted in a death rate of 37% for the 157 untreated animals that were given implants. At necropsy, 100% of untreated animals and 34 (59%) of 58 chloramphenicol recipients had intraabdominal abscesses. These results were unexpected since chloramphenicol showed excellent activity in vitro against the major isolates recovered from infected sites in untreated animals that had received the same challenge. The MICs of chloramphenicol for these organisms were 8 µg/ml for B. fragilis, 4 µg/ml for F. varium, and 4 µg/ml for E. coli. The dosage of chloramphenicol given to the rats resulted in a mean peak concentration in their sera of 22 µg/ml, a level well above the MIC values. This discrepancy between in vitro and in vivo results suggested the possibility that obligate anaerobes might be inactivating chloramphenicol in vivo.

In preliminary tests of the ability of obligate anaerobes to inactivate chloramphenicol, known concentrations of this antimicrobial agent were added to 18-hr. broth cultures of the test microorganisms diluted to  $10^8$  cfu/ml. The results (table 1) indicate that both B. fragilis (ATCC 23745) and C. perfringens (249) were capable of inactivating chloramphenicol over an incubation period of 6 hr. B. fragilis decreased the concentration of biologically active chloramphenicol from 97 µg/ml to 39 µg/ml during this incubation period, while C. perfringens decreased the concentration from 100 µg/ml to 2 µg/ml over the same interval.

Table 1 Detection of chloramphenicol and its aminophenyl derivative in bacterial culture after the addition of chloramphenicol.

Species, hr after addition of chloramphenicol	Detected in cultures	
	Chloramphenicol (µg/ml)	Aminophenyl derivative* (µg/ml)
<i>Bacteroides fragilis</i>		
0	97	0
3	72	25
6	39	50
<i>Clostridium perfringens</i>		
0	100	0
3	65	35
6	2	63

\*D-threo-1-(p-aminophenyl)-2-dichloroacetamido-1,3-propanediol.

Additional testing showed that filtrates of the culture broth of these strains also inactivated chloramphenicol. The strains used in these studies did not develop in vitro resistance to the antimicrobial agent after exposure. The inactivation of chloramphenicol proved to be dependent on the size of the inoculum: similar tests with  $10^6$  cfu of bacteria/ml failed to decrease the concentration of active drug. The ability of B. fragilis to inactivate chloramphenicol was tested with 19 additional bacterial strains obtained from clinical sources. All 19 strains inactivated the antimicrobial agent, with a mean decrease of 60% of active chloramphenicol after incubation for 6 hr. Since in vitro resistance to the drug did not develop in these strains, it was considered unlikely that acetylation was responsible for inactivation of the antimicrobial agent. A quantitative assay for other inactive derivatives of chloramphenicol was necessary to define the mechanism of inactivation.

Analysis by gas-liquid chromatography of silyl derivatives of chloramphenicol and related compounds revealed the presence of the amino phenyl derivative of chloramphenicol in bacterial cultures exposed to this agent (table 1). Quantitative determinations of the concentration of the inactive aminophenyl compound suggest, as does the absence of other derivatives a direct reduction of the nitro group of chloramphenicol to its corresponding amine. Recent data also indicate that the inactive aminophenyl derivative can be detected in the purulent exudate obtained from infected rats given chloramphenicol. This suggests that the discrepancy between in vivo and in vitro results may be explained by the reduction of chloramphenicol by anaerobes.

B. Reduction of mortality by metronidazole. Metronidazole was administered to 50 rats implanted with cecal inoculum. The results showed a significant reduction in early lethality; the death rate of treated animals was only 10%, in contrast to a rate of 37% in untreated control animals (table 2). This decrease was unexpected since our previous studies with this model showed that coliforms were the primary cause of bacteremia and lethality in the initial stages of infection. Studies of susceptibility in vitro showed that the MIC of metronidazole for E. coli cultured from the blood of untreated animals was 1,024 µg/ml, a concentration well above the peak serum level of 30 µg/ml in treated rats. As expected, there was a reduction in the number of abscesses seen at necropsy, and the strain of B. fragilis used in this experiment was highly susceptible to metronidazole (MIC=0.5 µg/ml). These results suggested two possibilities: that metronidazole has some effect on E. coli in vivo that was not consistent with the in vitro effect indicated by the susceptibility data, or that in this model anaerobes made a more significant contribution to early lethality than had been previously realized.

Additional animals were implanted with either E. coli (BVA 1-13) alone or a combination of E. coli and B. fragilis (ATCC 23745). All inocula contained a total of  $1 \times 10^8$  cfu/ml, with equal numbers of E. coli and B. fragilis used in the mixed inocula. It was found that the animals that received only E. coli with and without metronidazole treatment, has death rates of 85% and 100%, respectively (table 2).

Table 2 Results of metronidazole treatment of rats given various inocula by ip implantation.

Inoculum, treatment*	Mortality (%)	No. of survivors with abscess (%)	Positive blood culture 24 hr after implant†
Cecal contents			
-	58/157 (37)	99/99 (100)	20/20
+	5/50 (10)	6/45 (13)	1/10
<i>Escherichia coli</i>			
-	10/10 (100)	...	10/10
+	17/20 (85)	0/3	10/10
<i>E. coli</i> and <i>Bacteroides fragilis</i>			
-	10/10 (100)	...	10/10
+	2/10 (20)	0/8	0/10

\*Untreated control = (-); metronidazole-treated = (+).

†Blood was obtained from animals not included in mortality study.

All animals tested had blood cultures that were positive for E. coli 24 hr. after implantation. (It should be noted that blood for cultures were obtained from animals that were not included in the mortality studies, since transthoracic puncture may contribute to lethal outcome).

In contrast to the animals that received E. coli alone, the recipients of a combination of E. coli and B. fragilis were markedly affected by treatment with metronidazole. Animals treated with metronidazole had a death rate of 20%, with none of 10 blood cultures positive for either organism. Untreated recipients of the combination of E. coli and B. fragilis had a 100% death rate, with all 10 blood cultures positive for E. coli. These data suggest that metronidazole has an effect on mortality only if anaerobes are part of the infecting inoculum. To exclude the possibility that anaerobes contribute to lethality in this model, rats were challenged with combinations of B. fragilis and E. coli in various concentrations. These studies showed that mortality was directly correlated with the size of the inoculum of E. coli; the addition of B. fragilis to the inoculum had no apparent effect.

Chemostat cultures of E. coli alone and of E. coli in combination with B. fragilis were employed to determine the effect of metronidazole on growth of E. coli that was not detectable by other in vitro systems. It was found that the population density of E. coli was not affected by a continuously increasing concentration of metronidazole (table 3).

Table 3 Effect of metronidazole on pure and mixed continuous cultures of *Escherichia coli* and *Bacteroides fragilis*.

Culture, time*	Log <sub>10</sub> cfu of		Metron- idazole (μg/ml)†
	<i>E. coli</i> /ml	<i>B. fragilis</i> /ml	
<i>E. coli</i>			
0	8.4	...	0
1	8.3	...	1.5
3	8.4	...	6.0
5	8.4	...	11.0
7	8.4	...	14.5
12	8.3	...	22.5
<i>E. coli</i> + <i>B. fragilis</i>			
0	9.0	8.4	0
1	8.6	7.5	0
3	8.3	6.9	0
5	7.8	6.1	0.2
7	7.9	5.7	0.8
12	8.3	5.5	2.0

\*Time (hr) after metronidazole was introduced into chemostat via the nutrient reservoir.

†Concentration of biologically active metronidazole.

In addition, the concentrations of metronidazole measured in the fermentation vessel corresponded closely to the calculated amounts that had been added via the nutrient reservoir. Since metronidazole is reduced by sensitive bacterial cells to a biologically active but labile compound that can no longer be detected by bioassay, the correspondence between the amount of metronidazole added to the culture and that found in the culture vessel indicated that the nitro group on the imidazole ring had not been reduced and had not, therefore, been activated by E. coli alone.

The effect of metronidazole on mixed cultures of E. coli and B. fragilis was very different. When metronidazole was added to these cultures, the E. coli population decreased from  $10^{9.0}$  to  $10^{7.8}$  cfu/ml after 5 hr. During this same interval, the population of B. fragilis decreased from  $10^{8.4}$  to  $10^{6.1}$  cfu/ml. In contrast to results of the previous experiment, no metronidazole was detected



in the fermentation vessel until the population of *B. fragilis* had decreased by more than 100-fold. These data suggest that metronidazole is reduced in the presence of *B. fragilis* to a form active against *E. coli*. Once *B. fragilis* has been eliminated from the fermentor or its concentration is sufficiently low, metronidazole is no longer reduced and the concentration of *E. coli* returns to its previous level. Since the rapid decrease in the level of *B. fragilis* could conceivably affect the *E. coli* population by mechanisms independent of changes in antimicrobial activity, a similar experiment was performed with clindamycin instead of metronidazole. No decrease in *E. coli* populations was seen despite a rapid decrease in concentrations of *B. fragilis*.

C. Development of a model simulating wound abscess. In initial experiments rats were injected sc, directly beneath a surgical incision, with the cecal inoculum and barium sulfate. It was found that within five days animals implanted in this manner developed discrete abscesses that became progressively larger until days 14-17 (table 4).

**Table 4** Results of implantation of various inocula into rats through a skin incision.

Inoculum	Result*		
	Death	Abscess	Positive blood culture
Cecal contents + BaSO <sub>4</sub>	0/10	10/10	1/10†
Cecal contents alone	0/10	10/10	0/10
Broth cultures			
<i>Bacteroides fragilis</i>	0/10	0/10	0/10
<i>Bacteroides melaninogenicus</i>	0/10	0/10	0/10
<i>Escherichia coli</i>	0/5	0/5	0/5

\*Results are expressed as number with given result/number tested.

†Culture was positive for *E. coli*.

In six animals tested the mean Eh of the purulent material measured in situ on day 7 was -113 mV, a result which indicated a reduced environment. Between the 14th and 17th days after challenge, the abscess drained spontaneously through the site of the incision, and in some cases, through the sinus tracts 2-3 cm from the incision. In contrast to the previously described model of intraabdominal sepsis, none of these animals died. Cultures of blood obtained 24 hr. after challenge were positive for only one of 10 animals, with *E. coli* being the only bacterial isolate. Cultures of abscess pus were uniformly positive for *E. coli*, enterococci, and *B. fragilis*. In this model cecal contents implanted without barium sulfate produced abscesses in all 10 recipients, a result different from that obtained by the intraabdominal sepsis model.

Implants of pure cultures of *B. fragilis* (ATCC 23745), *B. melaninogenicus* (BCH 10946), or *E. coli* (BVA 1-13), each at a concentration of  $5 \times 10^7$  cfu/ml, failed to cause abscesses in the model of soft tissue infection (table 4). However, implants of the same size inoculum of *B. fragilis* combined with 50% vol/vol sterile cecal contents resulted in the formation of abscesses in all 15 recipients (table 5).

Table 5 Results of implantation of various *Bacteroides* species into rats through a skin incision.

Inoculum	Result	
	Death*	Abscess†
<i>Bacteroides fragilis</i>	0/15	15/15 (6.0)
<i>B. fragilis</i> after immunization‡	0/5	5/5 (6.0)
<i>Bacteroides distasonis</i>	0/5	0/5
<i>Bacteroides thetaiotaomicron</i>	0/5	3/5 (3.2)
<i>Bacteroides vulgatus</i>	0/5	3/5 (4.0)
<i>Bacteroides asaccharolyticus</i>	0/15	15/15
<i>Bacteroides melaninogenicus</i> subspecies <i>intermedius</i>	0/10	10/10

NOTE. All cultures contained  $5 \times 10^7$  cfu of bacteria and 50% (vol/vol) sterile cecal contents.

\*Number that died/number tested.

†Number with abscess/number tested (mean diameter of abscess in mm).

‡Animals immunized prior to implantation with the capsular polysaccharide of the homologous strain of *B. fragilis*.

Interestingly, animals that had been immunized prior to implantation with the capsular polysaccharide of the implanted strain of *B. fragilis* (1) also developed abscesses. These data suggest that circulating antibody cannot prevent abscess formation or eliminate *B. fragilis* from an abscess during wound infection. Implants of other closely related *Bacteroides* species in combination with 50% vol/vol sterile cecal contents produced abscess formation in six of 15 animals (table 5).

Additional studies have been done using inocula composed of 50% (vol/vol) sterile cecal contents combined with *B. asaccharolyticus* (BCH 382 and 536). This organism was of particular interest since a capsular polysaccharide has recently been isolated from it (2). All 15 animals implanted with this inoculum developed abscesses. In addition, all 10 animals implanted with *B. melaninogenicus* subspecies *intermedius* (BCH 10946 and BVA 1-21), a species that is phenotypically similar, also developed abscesses. These data indicate that, in this model, subcutaneous abscess is potentiated by a number of species of *Bacteroides*.

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